HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2011 July 01.

Published in final edited form as:

Nat Med. 2011 January ; 17(1): 117–122. doi:10.1038/nm.2261.

Podocyte secreted Angiopoietin-like 4 mediates proteinuria in glucocorticoid sensitive nephrotic syndrome

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The major manifestations of nephrotic syndrome include proteinuria, hypoalbuminemia, edema, hyperlipidemia and lipiduria. Common causes of nephrotic syndrome are diabetic nephropathy, minimal change disease (MCD), focal and segmental glomerulosclerosis (FSGS), and membranous nephropathy (MN). Among the primary glomerular diseases, MCD is usually glucocorticoid sensitive, whereas the response in other diseases is variable (1). Despite recent identification of key structural proteins in the glomerular capillary loop, many disease mechanisms in nephrotic syndrome remain unresolved. In this study, we show that the glomerular expression of Angiopoietin-like 4 (Angptl4), a secreted glycoprotein, is glucocorticoid sensitive, and is highly upregulated in podocytes in experimental and human MCD. Podocyte-specific transgenic overexpression (NPHS2-*Angptl4*) in rats induces nephrotic range selective proteinuria (500 - fold increase in albuminuria), loss of GBM

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Lionel C. Clement: Lead postdoctoral fellow in this study, conducted the majority of the animal studies, generated stable cell lines and purified recombinant protein, maintained and conducted all studies on transgenic rats, all confocal imaging and in situ hybridization and most gene expression studies. Also did selected 2D gel studies.

Carmen Avila Casado: Interpreted and analyzed light microscopy, electron microscopy and immunogold electron microscopy sections for the study. Conducted studies on induction of collapsing glomerulopathy in rats.

Camille Macé: Postdoctoral fellow, conducted the majority of 2D gel electrophoresis and proteomic work, and most of the albumin ELISA assays.

Elizabeth Soria: Electron microscopist and morphometrics expert, prepared tissue for electron microscopy, conducted and imaged conventional and most immunogold electron microscopy studies, conducted alcian blue charge studies.

Winston W. Bakker: Provided human sera from his previously published studies on minimal change disease, assisted in study design and preparation of manuscript.

Sander Kersten: Conducted studies on Angptl4 transgenic mice, and provided tissue for histological and gene expression analysis, conducted *Angptl4*$^{-/-}$ mouse studies with NTS, made very substantial contributions towards the preparation of the manuscript.

Sumant S. Chugh: Senior Investigator, planned and supervised the entire study, generated constructs for transgenic rats, conducted several molecular biology and gene expression studies, conducted all early animal studies, wrote and revised the manuscript with input from co-authors.

charge and foot process effacement, whereas adipose tissue-specific transgenic expression (aP2-*Angptl4*) results in increased circulating Angptl4 levels, but no proteinuria. *Angptl4*$^{-/-}$ mice injected with lipopolysaccharide or nephritogenic antisera develop significantly lower proteinuria than controls. Angptl4 secreted from podocytes in some forms of nephrotic syndrome lacks normal sialylation, and feeding sialic acid precursor N-Acety-D-mannosamine (ManNAc) to NPHS2-*Angptl4* transgenic rats increases sialylation of Angptl4 and reduces albuminuria by over 40%. These studies suggest a key role of podocyte secreted Angptl4 in nephrotic syndrome.

As part of our studies to identify novel mechanisms of proteinuria, we injected rats with the γ2 fraction of nephtotoxic serum (γ2-NTS), an antiserum reactive to multiple podocyte proteins that induces complement- and leukocyte-independent glomerular injury by direct antibody binding (2). A panel of differentially expressed glomerular genes was assembled (3), and two genes not previously known to be involved in the pathogenesis of proteinuria investigated in detail. One of these genes encodes for the transcriptional factor zinc fingers and homeoboxes 3 (*Zhx3*) expressed in podocytes, and has now been shown to play a significant role in the pathogenesis of primary glomerular disease (3). The other gene, Angptl4, was highly upregulated in the podocyte, and is the focus of investigation in this paper.

Angiopoietin-like proteins have been implicated in the development of hypertriglyceridemia (4) and tumor metastasis (5), and have some functional properties that are different from the angiopoietins. Angptl4 is a *Pparγ* (6) and *Pparα* (7) target gene highly expressed in the liver and adipose tissue, strongly induced by fasting in white adipose tissue and liver, and is an apoptosis survival factor for vascular endothelial cells under normoxic conditions (8). Angptl4 is a potent inhibitor of lipoprotein lipase (LPL) (4), and induces significant hypertriglyceridemia following intravenous injection or adenovirus-mediated expression (9, 10). Other studies show lesser expression in cardiomyocytes and skeletal muscle, and low level expression in whole kidney on Northern blot analysis (6). Recent population based studies of the *ANGPTL4* gene reveal variants that affect triglyceride levels in humans (11, 12). Most normal circulating Angptl4 in rodents is secreted from the liver as a cleaved protein that binds to HDL particles (13). A role of Angptl4 in proteinuria has not been previously reported.

We noted severe upregulation (70.45 ± 4.14, mean ± SEM) of *Angptl4* mRNA in rat glomeruli at the peak of complement- and leukocyte-independent heterologous phase proteinuria 24 hours after injection of γ2-NTS (Fig. 1a, b, Supplementary Fig. 1). Injection of *Angptl4*$^{-/-}$ and *Angptl4*$^{+/+}$ mice with LPS (Fig. 1c) and γ2-NTS (Supplementary Fig. 2a,b) induced significantly less proteinuria (Fig. 1c, Supplementary Fig. 2a) and foot process effacement (Supplementary Fig. 2b) in *Angptl4*$^{-/-}$ mice. Normal rat glomeruli express Angptl4 in a capillary loop pattern that co-localizes with podocyte protein CD2 adapter protein (CD2AP) (Fig. 1d). We noted early (Day 3, before the onset of proteinuria) and progressive upregulation of *Angptl4* mRNA expression in young rats following intravenous injection of a single dose of puromycin aminonucleoside (PAN model, peak up to 80-fold increase in different studies) (Fig. 1e), and *in situ* hybridization confirmed upregulation in a peripheral capillary loop pattern (Supplementary Fig. 2c). In passive Heymann nephritis, we

saw a smaller increase in *Angptl4* expression, starting after the onset of proteinuria (Fig. 1e). *Angptl4* mRNA expression did not change in anti-Thy1.1 nephritis, or in collapsing focal and segmental glomerulosclerosis (FSGS) (Fig. 1e) (14). Angptl4 protein expression increased dramatically in podocytes (Fig. 1f) following induction of PAN, with substantial additional overlap with the GBM, and this was confirmed by immunogold electron microscopy (EM) (Fig. 1g). We also noted reduced expression of GBM heparan sulfate proteoglycans (HSPG) in PAN glomeruli (Fig. 1f).

To study the biological role of *Angptl4* upregulation in nephrotic syndrome, we first assessed Angptl4 expression in the podocyte in a previously published *Angptl4* transgenic mouse model (13) (Fig. 2a–2d, Supplementary Fig. 2d,e). Glomeruli appeared normal on light microscopy (Fig. 2a). We noted increased glomerular expression of Angptl4, but not of another podocyte expressed protein Zona Occludens 1 (ZO-1) (Fig. 2a) in 3 month old transgenic mice. Angptl4 expression co-localized with podocyte expressed CD2AP (Supplementary Fig. 2d). Electron microscopy revealed 50% effacement or broadening of foot processes (Fig. 2b). immunogold electron microscopy revealed a large number of gold particles in podocyte foot processes, GBM and close to the endothelial cell surface (Fig. 2c), especially in areas opposite to foot process effacement (Fig. 2c, **right panel**). These mice had mild proteinuria (Fig. 2d).

To study higher levels of Angptl4 upregulation noted in disease states, we generated transgenic rat models, one each for podocyte specific (NPHS2-*Angptl4*) and adipose tissue specific (aP2-*Angptl4*) transgenic over expression (Fig. 2e). Analysis of mRNA expression in organs that normally express *Angptl4* confirmed specificity of expression (Fig. 2f, Supplementary Fig. 2f). In keeping with the normal expression pattern of the *NPHS2* promoter, we noted significant expression in the pancreas. Histological assessment of 3 month old heterozygous male NPHS2-*Angptl4* transgenic rats revealed normal appearing glomeruli with prominent podocytes on light microscopy (Fig. 2g) and increased glomerular Angptl4 expression by confocal imaging (Fig. 2h) that overlaps with podocyte nephrin and GBM heparan sulfate proteoglycans (HSPG). Electron microscopy of 5 month old homozygous TG rats revealed diffuse foot process effacement (Fig. 2i, Supplementary Fig. 3a). Using immunogold electron microscopy in homozygous NPHS2-*Angptl4* transgenic rats, we observed a correlation between gold particle clustering in the GBM and the development of foot process effacement, which is first noted around age 3 months (Fig. 2j). *In situ* hybridization for *Angptl4* (Supplementary Fig. 3b) revealed selective increase in the signal in glomeruli in a peripheral podocyte distribution. The aP2-*Angptl4* transgenic rats had morphologically normal glomeruli by light (Fig. 2g) and electron microscopy (not shown).

We noted significant albuminuria in both NPHS2-*Angptl4* TG rat founder lines. Female homozygous and male heterozygous rats developed albuminuria at age 1 month (Fig. 3a–3c, Supplementary Fig. 3c). Homozygous females developed up to 100-fold, heterozygous males up to 20-fold and homozygous males over 500-fold increase in albuminuria over time. Heterozygous females were not albuminuric. Over 90% of the urinary protein comprises of intact albumin (Fig. 3d, Supplementary Fig. 3d), thereby making these rats the first model of selective proteinuria. Blood pressure was significantly lower in proteinuric heterozygous

NPHS2-*Angptl4* transgenic rats compared to wild type controls (Supplementary Fig. 3e). By contrast, aP2-Angptl4 transgenic rats did not develop albuminuria (Fig. 3d), despite high circulating Angptl4 levels (Supplementary Fig. 4a–4d). Despite upregulation of *Angptl4* in pancreas in NPHS2-*Angptl4* transgenic rats, circulating Angptl4 levels were not elevated, and were in fact marginally reduced (Supplementary Fig. 4a–4d) due to presence of proteinuria in these rats. In keeping with the proteinuric effects of podocyte secreted Angptl4, NPHS2-*Angptl4* transgenic rats developed more proteinuria (Fig. 3e) than wild type littermates after induction of PAN. Serum electrolytes, creatinine and glucose levels were indistinguishable from wild type rats.

As also previously published (15), proteinuria in PAN is partially glucocorticoid sensitive on day 6 (Fig. 3f). mRNA expression studies for Angptl4 revealed significant reduction in *Angptl4* expression in glucocorticoid treated rats on day 6 (Fig. 3g), thereby suggesting that *Angptl4* was a glucocorticoid sensitive gene in vivo. Early increase in *Angptl4* mRNA expression noted on PAN Day 3 (before the onset of proteinuria) was also reversed in glucocorticoid treated rats. In addition, Angptl4 oligomers were also detectable in the urine in experimental models of proteinuria (Supplementary Fig. 4e,f).

We investigated loss of GBM charge in transgenic rats and mice (Supplementary Fig. 5). NPHS2-*Angptl4* transgenic rats had significant loss of GBM charge by alcian blue and polyethyleneimine methods (Supplementary Fig. 5a,b,d). As also seen in PAN rats (Fig. 1f), GBM heparan sulfate proteoglycan is also reduced in NPHS2-*Angptl4* transgenic rats (Supplementary Fig. 5c).

Immunoblotting of glomerular proteins typically underestimates Angptl4 production, since the protein is rapidly secreted. On 2D gel electrophoresis, most Angptl4 in normal glomeruli migrated as glycosylated low order oligomers at neutral pI (Fig. 4a), though less prominent spots for both glycosylated intact 70 KDa and cleaved / non glycosylated 45 KDa monomeric forms were also noted (Supplementary Fig. 6a,b). The 45 kDa protein, but not the oligomers, were threonine phosphorylated (Supplementary Fig. 6a). In PAN, both neutral pI oligomers, that were reactive with sialic acid binding lectin Maackia amurensis (MAA), and high pI oligomers, that were not MAA reactive, were increased (Fig. 4a,b). This increase was blunted in PAN treated with glucocorticoids, though disproportionately higher amounts of high pI oligomers were noted (Fig. 4a,b).

Next, we studied the sialylation of Angptl4 (Fig. 4, Supplementary Fig. 6) in vitro (Fig. 4c, d, Supplementary Fig. 6c,d) using the *Angptl4*-HEK293 (Supplementary Fig. 7a–7c) and *Angptl4*-GEC stable cell lines (Supplementary Fig. 7d,e). Incubation of both stable cell lines with sialic acid precursor ManNAc resulted in a shift of the secreted Angptl4 protein towards neutral pI, with increased reactivity to MAA, thereby suggesting that sialylation played a significant role in the differential electrophoretic migration of Angptl4 (Fig. 4c,d).

Since an increase in hyposialylated Angptl4 was noted in PAN, a potential role of this lack of sialylation in the pathogenesis of proteinuria was studied. Over half of glomerular Angptl4 in NPHS2-Angptl4 transgenic rats was hyposialylated and migrated at a high pI (Fig. 4e,f, Supplementary Fig. 6e). NPHS2-*Angptl4* transgenic rats that received ManNAc in

tap water had reduced albuminuria (Fig. 4g, Supplementary Fig. 8a,b), with 40.6 ± 3.3% (mean ± SEM) reduction in albuminuria over a 12 day period compared to baseline, and returned to levels comparable with control untreated transgenic rats after 24 days of washout. Densitometry analysis of Western blots for glomerular Angptl4 from rats euthanized on Day 12 (Fig. 4f) showed an increase in the neutral pI Angptl4 fraction from 43.3 ± 4.2% (mean ± SEM) in control rats to 72.9 ± 1.4% (mean ± SEM) in ManNAc treated rats. The neutral pI fraction was also reactive with the sialic acid binding lectin Sambucus nigra (SNA I) (Fig. 4e), confirming increased sialylation of Angptl4 in ManNAc treated rats. We did not note any changes in the quantitative expression of podocalyxin, another sialylated glomerular glycoprotein important in podocyte structural integrity, or significant shifts towards a lower pI (below 7) in ManNAc treated TG rats (Supplementary Fig. 6f–h).

In normal rodents, circulating Angptl4 is mostly HDL bound cleaved protein secreted by the liver (13). Analysis of oligomer formation by Angptl4 in transgenic rats showed that most glomerular Angptl4 was either monomeric or formed low order oligomers, whereas circulating Angptl4 formed mostly middle order and high order oligomers (Supplementary Fig. 8c). This may be one reason why the circulating form does not enter the GBM in aP2-*Angptl4* transgenic rats. Most circulating Angptl4 in these rats migrates between pI 5 and 7 (Supplementary Fig. 4a,b), and is therefore unlikely to avidly bind anionic GBM proteins in a charge dependant manner.

Of the various forms of primary glomerular disease examined, *Angptl4* expression was most significantly upregulated in experimental MCD, less prominently and later in the disease course in experimental membranous nephropathy, and was unchanged in experimental FSGS. Therefore, Angptl4 expression was studied in biopsies from individuals with MCD by confocal imaging (Supplementary Fig. 9). Increased expression in a podocyte distribution with additional GBM overlap was noted in all 5 biopsies. In addition, Angptl4 oligomers were detectable in the urine of an additional four individuals with MCD (Supplementary Fig. 10a,b, Supplementary Table 1), and distinctive patterns of circulating Angptl4 expression were noted in their plasma (Supplementary Fig. 10c–10e).

The effects of podocyte secreted Angptl4 on the glomerular capillary loop in nephrotic syndrome are illustrated in Supplementary Fig. 11. Transgenic expression of *Angptl4* from the podocyte reproduced some of the key features of human MCD, including the classic morphologic changes, nephrotic range selective proteinuria, and loss of GBM charge. One feature that is prominently missing is the explosive onset of proteinuria. It is possible that this feature is contributed to by other genes / proteins concomitantly dysregulated with *Angptl4* in human MCD, and that these putative proteins may accelerate the interaction or transport of Angptl4 across the glomerular capillary loop. These results demonstrate that Angptl4 may be involved in the pathogenesis of MCD. Development of albuminuria in NPHS2-*Angptl4* transgenic rats at age 1 month, when podocyte foot processes are intact and scattered immunogold particles are noted in the GBM, suggests that podocyte secreted Angptl4 induces a filtration defect in the GBM. Clustering of immunogold particles in the GBM coincides with the development of foot process effacement and a sharp increase in

proteinuria around age 3 months, suggesting a complex interaction between Angptl4, GBM proteins and the podocyte – GBM interface.

Glucocorticoid sensitivity of *Angptl4* in PAN favors a significant role for this gene in glucocorticoid sensitive nephrotic syndrome. Other podocyte expressed glucocorticoid sensitive genes have been described in literature (15). Mutations in one of these genes, *NPHS2*, most often results in the development of glucocorticoid resistant FSGS (16). Of proteins secreted by the podocyte, published studies suggest a role of *VEGFA* in human thrombotic microangiopathy (17), whereas experimental overexpression of *Angpt2* in mice results in a 2 – 2.5 fold increase in albuminuria (18). Neither of these genes is known to be directly involved in the pathogenesis of glucocorticoid sensitive nephrotic syndrome.

Absence of proteinuria in aP2-*Angptl4* transgenic rats, despite high circulating Angptl4 levels, highlights the importance of production of Angptl4 in the podocyte in proteinuric disease. Also, the commonly observed 60 to 80 fold peak upregulation of glomerular *Angptl4* expression in PAN is close to the 120-fold increase in *Angptl4* expression noted in proteinuric heterozygous male NPHS2-*Angptl4* transgenic rats, thereby making these rats a suitable model for future studies on nephrotic syndrome.

The secretion of high pI Angptl4 by the podocyte in PAN is likely to facilitate the tethering of Angptl4 to the GBM, and prior studies have shown the binding of Angptl4 to heparan sulfate proteoglycans (19). This correlates well with reduced GBM charge in rodents with transgenic expression of Angptl4 from the podocyte. A relationship of GBM charge with proteinuria is hard to conclude from these studies, since despite mild proteinuria in the transgenic mouse model, the reduction in GBM charge is comparable between the *Angptl4* transgenic mouse and NPHS2-*Angptl4* transgenic rat models. It is likely that charge facilitates the transit of high pI Angptl4 across the GBM against the direction of fluid flow.

Changes in sialylation may explain some of the variations in pI, since increasing the sialylation of Angptl4 in vitro induces migration from high towards neutral pI. There are at least two potential O-glycosylation sites in rat and human Angptl4 where sialic acid residues could be incorporated. The reduction in albuminuria in NPHS2-*Angptl4* transgenic rats fed with ManNAc, and increase in the sialylation of glomerular Angptl4 suggest that hyposialylation may be one mechanism by which Angptl4 overexpression contributes to proteinuria. Therefore, treatment with sialic acid precursors constitute a potential therapeutic tool to reduce proteinuria in some forms of nephrotic syndrome. ManNAc therapy has been used recently to improve muscle weakness (20) and the severe glomerular phenotype (21) in mice harboring mutations in the GNE/MNK gene seen in patients with hereditary inclusion body myopathy. The kidney phenotype noted (21) however does not have a counterpart in humans with this disease.

The introduction of Angptl4 in the field of proteinuria investigation is likely to stimulate additional studies of its role in other aspects of nephrotic syndrome. A recent publication (22) suggests that glucocorticoids increase Angptl4 expression in adipose tissue, and could potentially increase levels in the circulation. Circulating Angptl4 increases plasma triglyceride levels by inhibiting tissue uptake via inactivation of endothelial bound LPL

activity in target organs (4). Future studies will address whether some of the hypertriglyceridemia in nephrotic syndrome is contributed to by Angptl4 leaking from glomeruli into the circulation. It would be equally important to study the interaction of Angptl4 with specific GBM proteins, and identify DNA motifs in the Angptl4 gene that mediate glucocorticoid sensitivity.

Methods

Generation of transgenic rats

We generated podocyte specific NPHS2-*Angptl4* transgenic rats in the following manner. We digested the vector pTRE-tight with StuI and EcoRI to remove the minimum CMV promoter between bp 278 and 324, blunt ended the 5' overhangs with T4 DNA polymerase, and re-ligated to generate pTRE-tight MP (minus promoter). For podocyte specific expression, we placed a rat Angptl4 cDNA construct (including the signal sequence) with a C-terminal V5 tag between MluI and SalI upstream of the SV40 polyA tail. We cloned the human *NPHS2* promoter upstream by PCR between SacI and BamHI using a published human *NPHS2* promoter construct (GenBank: AF487463.1) as template without DMSO to exclude a naturally occurring loop between bps 2343 and 2568 to improve expression.

We generated the adipose tissue specific rat Angptl4 construct in the vector that contained the 5.4 Kb mouse aP2 promoter construct (27) (purchased from Addgene Inc. Cambridge MA USA) by cloning the rat *Angptl4* cDNA (including the signal sequence) with a C-terminal V5 tag at the NotI site just upstream of the polyA tail.

Transgenic rats were generated by microinjection of the digested DNA constructs into fertilized Sprague Dawley eggs (conducted at University of Michigan), implantation into pseudopregnant host Sprague Dawley females, and the resulting offsprings were genotyped by routine PCR and TaqMan genomic DNA real time PCR strategy using construct specific and control genomic prolactin primer and probe combinations (Supplementary Table 2). Two founder lines for podocyte specific expression, and 3 lines for adipose tissue specific expression were generated. Data from NPHS2-*Angptl4* transgenic rat line 740 (5 copies of the transgene) and aP2-*Angptl4* transgenic rat line 375 (3 copies), both stable over 6 generations, are presented. We studied total Angptl4 mRNA expression in multiple organs that normally express *Angptl4* (n = 3 rats / study). Urinary total protein was assessed (minimum 6 rats / group) using the Bradford method (Biorad laboratories, Hercules CA USA), and albuminuria by ELISA (Bethyl laboratories, Montgomery TX USA). For assessment of urinary protein by 7.5 % reducing SDS PAGE and comparison with human disease, 3 μg of protein was loaded per lane for each condition (except MCD remission), gels stained with GelCode blue (Thermo Fisher Scientific Inc. Waltham MA USA), and densitometry assessed for the whole lane and the 70 kDa band. Serum electrolytes, BUN, creatinine and glucose levels were assessed in transgenic rats and wild type littermates (minimum 6 rats / group) using a Alfa Wassermann Vetace autoanalyzer (Schiaparelli Biosystems Inc, Fairfield, NJ). Histological assessment was conducted in 3 rats / group.

For induction of PAN in heterozygous NPHS2-*Angptl4* transgenic rats or controls (n = 8 rays / group) by the single intravenous injection method, we used a lower dose of puromycin aminonucleoside (10 mg / 100 gm body weight; usual dose 15 mg / 100 gm body weight).

Development of *Angptl4* stable cell lines

We developed two stable cell lines using a rat *Angptl4* pcDNA 3.1-V5/His construct, along with control empty vector stable cell lines. The HEK293 *Angptl4* stable cell line secretes a 70 kDa protein in serum free conditions and a 55 kDa protein in the presence of serum. The full length V5-His tagged protein is affinity purified from serum free media using a Nickel affinity column. An immortalized mouse GEC stable cell line was similarly developed and secretes 70 kDa Angptl4 without tags, since mouse GECs cleave the V5 or other tags from all recombinant proteins (3). This protein was affinity purified using an anti-Angptl4 antibody column. The morphology of both stable cell lines was indistinguishable from their respective control stable cell lines or control cells.

In vitro studies with ManNAc

To study the effect of ManNAc on the sialylation of recombinant protein, we grew the *Angptl4*-HEK293 stable or pcDNA3.1-HEK293 control stable cell lines to confluence in 15 cm dishes, washed them twice with warm PBS, then incubated them with serum free DMEM without Phenol Red, with or without 25 mM ManNAc (Sigma Aldrich, St. Louis MO USA), for 48 hours, after which we harvested and concentrated the supernatant, assayed protein concentration, and resolved the protein by 2D gel electrophoresis (50 μg / gel). We conducted Western blot studies using rabbit anti-Angptl4 antibody, after which we stripped the blots and incubated them with lectin MAA-HRP (E-Y laboratories, San Mateo CA USA). Similarly, we grew the GEC-*Angptl4* and control stable cell lines at 33°C, washed them twice with warm PBS, incubated them Phenol Red free RPMI 1640 without serum, with or without ManNAc (25 mM), for 48 hours, harvested and concentrated the supernatant, measured protein concentration, and resolved the protein by 2D gel electrophoresis (200 μg / gel).

In vivo studies with ManNAc: We first conducted a pilot study in heavily albuminuric NPHS2-*Angptl4* rats. We added ManNAc to tap water at an initial dose of 1 mg / ml, and doubled the dose every 3 days immediately after an 18-hour urine collection. After 12 days, we stopped ManNAc, and measured albuminuria periodically over the next 12 days. This study revealed that moderate doses of ManNAc reduce albuminuria, and that this process is reversible.

To make the next study affordable, we used rats with lower baseline albuminuria (15 – 25 fold higher than wild type rats). We treated 7 homozygous male NPHS2-*Angptl4* transgenic rats (age 3 months) with 1 mg / ml ManNAc in tap water for 12 days (Treatment group, ManNAc phase), after which three rats were euthanized and the others returned to plain tap water for another 24 days (Treatment group, Washout phase). We did two 18 hour urine collections (Day -12 and Day 0) before the start of the study to confirm rising albuminuria. We assessed another 7 male NPHS2-*Angptl4* transgenic rats of similar age for baseline albuminuria, gave them normal tap water (control group), euthanized three rats on Day 12

and followed others up to Day 36, at which point we reassessed albuminuria and euthanized them. We measured daily water intake of rats in both groups. At the Day 12 euthanasia time point, we removed the kidneys, isolated glomeruli and processed them for 2D gel electrophoresis (n = 3 blots / condition). We denoted albuminuria on Day 0 for each rat as 100%, and expressed all subsequent albuminuria values relative to Day 0. Western blot studies were conducted as described for the in vitro studies.

Statistical analysis

Throughout the manuscript, values in graphs are mean ± SEM. Analysis of difference in proteinuria or gene expression involving three or more groups was conducted by ANOVA with post analysis testing using GraphPad InStat software, Version 3.05. For comparison of two groups, the unpaired Students t test in Microsoft Excel 2003 was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Reagents: Bruce Spiegelman (Dana-Farber Cancer Institute, mouse aP2 promoter construct), Lawrence Holzman (University of Michigan, human NPHS2 promoter construct), Peter Mundel (University of Miami, mouse GECs), Toin van Kuppevelt (Radboud University Nijmegen Medical Center, anti-HSPG antibody p1113), David Salant (Boston University, γ2-NTS), Anja Köster (Eli Lilly Corporation, Angptl4$^{-/-}$ mice).

Other contributions: Guangxing Bai PhD (generation of probe for *in situ* hybridization), Suresh Shastry PhD (some ELISA assays for rat, mouse albumin), Syed Rahmanuddin MD (selected immunogold studies, polyethyleneimine studies), Frits Mattijssen PhD (mouse LPS study), Jan Novak PhD (advice on choice of lectins), Yashpal S. Kanwar MD PhD and Manjeri Venkatachalam MD (useful discussions on heparan sulfate proteoglycans).

Funding: NIH grants R56DK077073, R01DK077073 and Norman S. Coplon Satellite Research Grant to SSC, CONACYT 111 grant to CAC.

Core facilities: UAB – UCSD George O'Brien Center Core C for measuring mouse urine creatinine by mass spectrometry.

Patents: The use of sialic acid precursors, including ManNAc, to treat proteinuria and nephrotic syndrome is covered by US Provisional Application 61/351,865 filed by Sumant S. Chugh.

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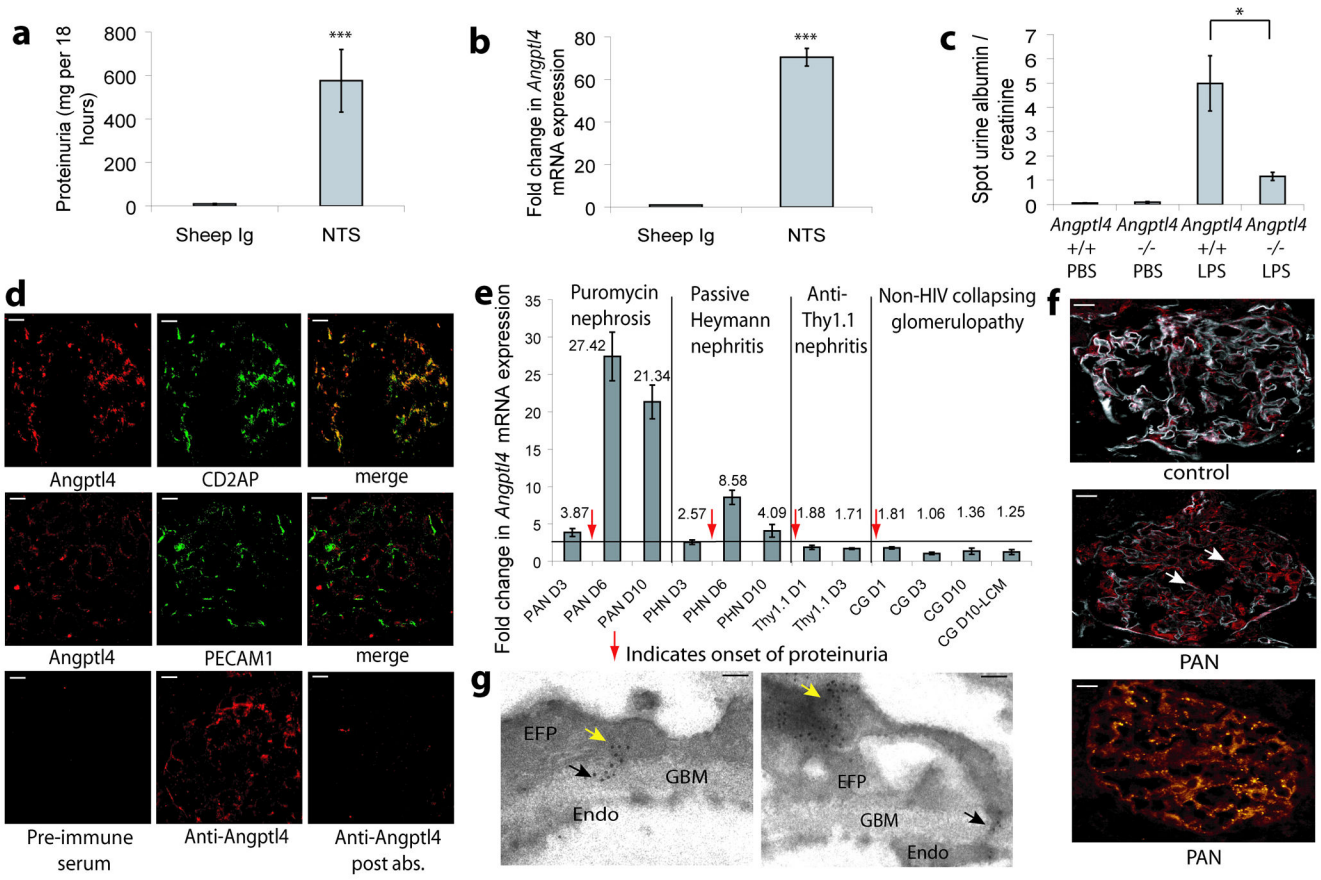

Figure 1.

Angptl4 mRNA and protein expression in experimental glomerular disease. (**a**) Induction of proteinuria in rats 24 hours after injection of γ2-NTS. (**b**) Upregulation of glomerular *Angptl4* mRNA expression in rats injected with γ2-NTS. (**c**) Proteinuria in *Angptl4*$^{-/-}$ and *Angptl4*$^{+/+}$ mice after injection of lipopolysaccharide (LPS). (**d**) Confocal expression of Angptl4 protein in rat glomeruli, and colocalization with podocyte protein CD2AP. Absorbing out reactivity from anti-Angptl4 antibody with recombinant Angptl4 abolished immunoreactivity. (**e**) Assessment of changes in glomerular *Angptl4* expression in rat models of minimal change disease (puromycin nephrosis, PAN), membranous nephropathy (passive Heymann nephritis, PHN), mesangial injury (anti-Thy1.1 nephritis), and severe focal and segmental glomerulosclerosis (non-HIV collapsing glomerulopathy, CG). Threshold for significance was 3-fold change. (**f**) Confocal assessment of Angptl4 expression (red) in control and PAN Day 6 glomeruli, and co-localization with GBM heparan sulfate proteoglycan (white, top and middle panels) and podocyte protein nephrin (green, overlap yellow, bottom panel). (**g**) Immunogold electron microscopy of PAN Day 6 rat glomeruli to demonstrate Angptl4 expression (gold particles) in the podocytes (yellow arrows) and GBM (black arrows). Scale bars (d) 7.5 μm (f) 10 μ(g) 0.33 μm. NTS (nephrotoxic serum), CD2AP (CD2 adapter protein), LCM (laser capture microdissection), GBM (glomerular basement membrane), EFP (effaced foot processes), Endo (endothelium).

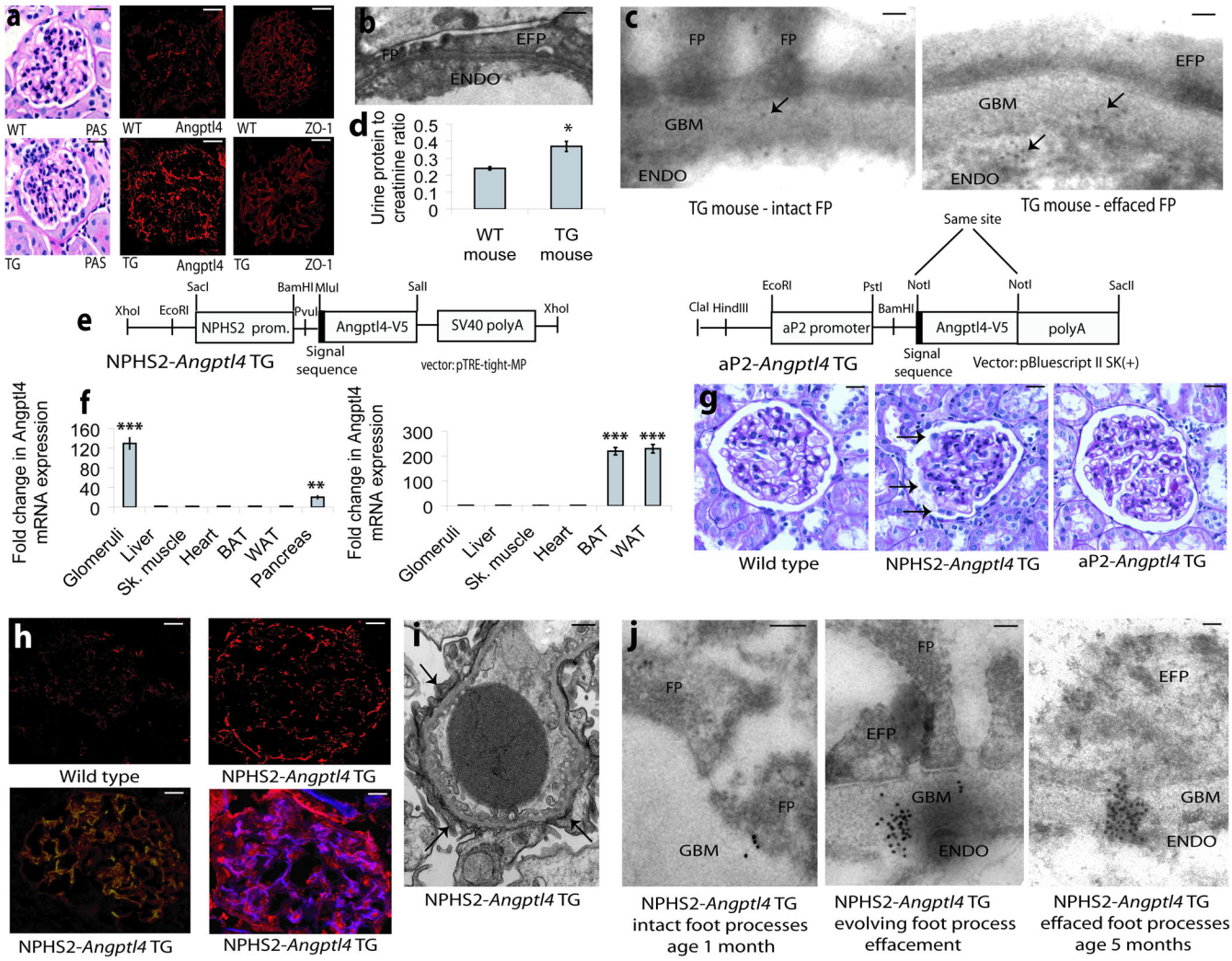

Figure 2.

Characterization of male *Angptl4* transgenic (TG) mice and rats. (**a**) Light microscopy (left panels) and confocal assessment of Angptl4 (middle panel) and ZO-1 (right panel) expression in *Angptl4* transgenic and wild type (WT) mice. (**b**) Electron micrograph of 3 month old transgenic mouse glomeruli, showing intact (FP) and effaced podocyte foot processes (EFP). (**c**) Immunogold electron microscopy for Angptl4 showing gold particles in podocytes and glomerular basement membrane (GBM, arrows) in *Angptl4* transgenic mice. (**d**) Proteinuria in 3 month old *Angptl4* transgenic mice. (**e**) Rat *Angptl4* transgenic constructs for the targeted expression of *Angptl4* in podocytes (NPHS2-*Angptl4*, left panel) and adipose tissue (aP2-*Angptl4*, right panel) in rats. (**f**) Multi-organ mRNA expression profile of *Angptl4* in podocyte specific and adipose tissue specific transgenic rats. (**g**) Periodic Acid Schiff stained sections from 3 month old wild type and heterozygous transgenic rats. Arrows point towards prominent podocytes in NPHS2-*Angptl4* transgenic rats. (**h**) Confocal expression of Angptl4 (red) in NPHS2-*Angptl4* transgenic rat glomeruli, and co-localization with podocyte protein nephrin (green, overlap yellow) and GBM heparan sulfate proteoglycan (blue, overlap fushia). (**i**) Electron micrograph of a glomerular

capillary loop from a 5 month homozygous NPHS2-*Angptl4* transgenic rat, showing diffuse foot process effacement (arrows). (**j**) Immunogold electron microscopy for Angptl4 in NPHS2-*Angptl4* transgenic rats of increasing age (left to right), with transition from intact foot processes to foot process effacement (first noted around age 3 months), and clustering of gold particles in the GBM noted prominently in areas opposite to effaced foot processes (middle and right panels).Scale bars (a) 10 μm (b) 1 μm (c) 0.25 μm (g) 10 μm (h) 8μm (i) 1 μm (j) 0.2 μm. ENDO (endothelium). ** $P < 0.01$, *** $P < 0.001$

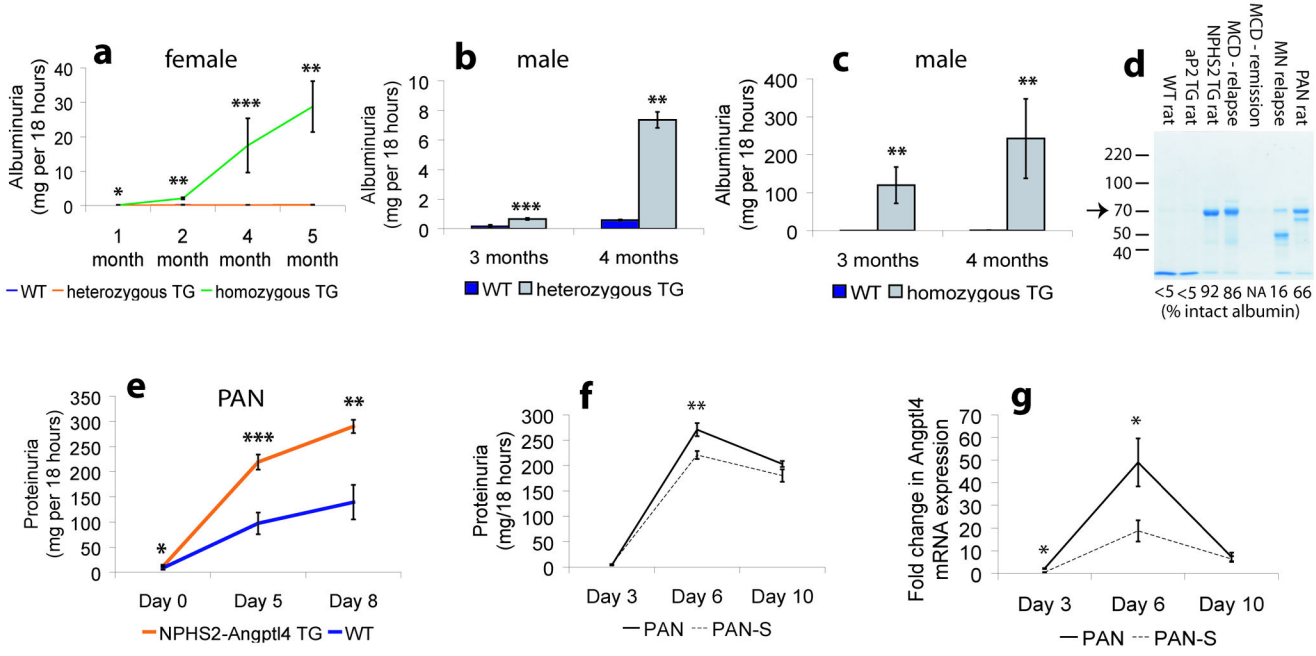

Figure 3.

Relationship of *Angptl4* overexpression with proteinuria. (**a**) Albuminuria in female NPHS2-*Angptl4* transgenic (TG) rats. (**b**) Albuminuria in male heterozygous NPHS2-*Angptl4* transgenic rats (**c**) Albuminuria in male homozygous NPHS2-*Angptl4* transgenic rats. (**d**) GelCode blue stained SDS PAGE of urinary protein from transgenic rats, rats with PAN, and individuals with minimal change disease (MCD) and membranous nephropathy (MN). Arrow points towards prominent 70 kDa intact albumin band. Mean percentage densitometry of intact albumin is shown for each lane (details in Supplementary Fig. 3d). (**e**) Proteinuria following induction of low dose PAN in heterozygous male NPHS2-*Angptl4* transgenic and wild type littermates. (**f**) Proteinuria in Wistar rats treated with glucocorticoids (PAN-S) or PBS (PAN) on alternate days starting 1 day after induction of PAN. (**g**) Glomerular Angptl4 mRNA expression in PAN rats described in panel f. * P<0.05; ** P < 0.01, *** P<0.001.

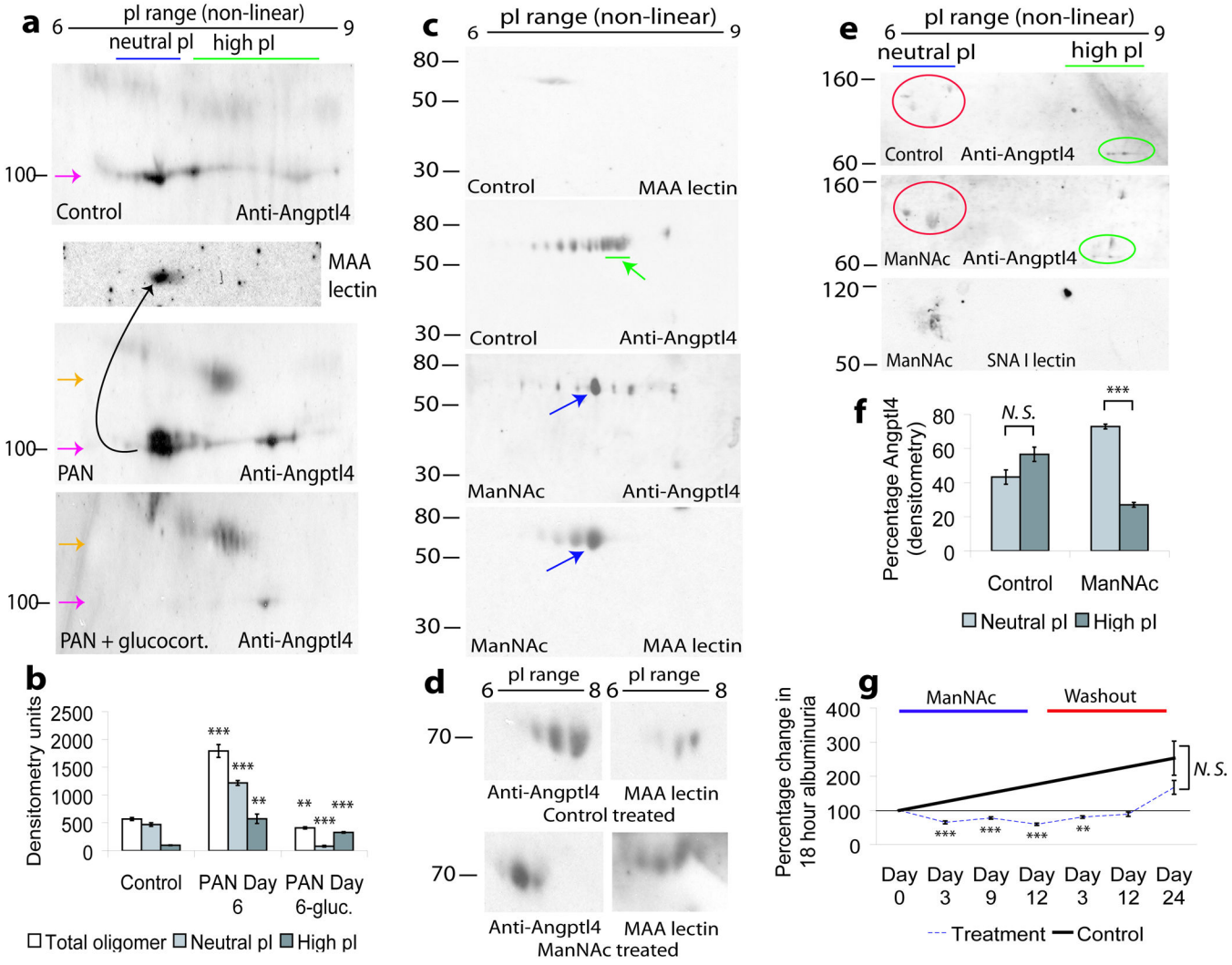

Figure 4.

Relationship of Angptl4 sialylation with proteinuria. (**a**) Two-dimensional (2D) gel electrophoresis and Western blot of protein from perfused glomeruli show neutral and high pI low order Angptl4 oligomers (pink, orange arrows) in control, PAN Day 6, and glucocorticoid treated PAN Day 6 rats (from experiment in Fig. 3f). Reactivity of sialic acid binding lectin MAA to these oligomers was also assessed (exemplified for PAN, excerpts from independent blots). (**b**) Densitometry of total, neutral and high pI oligomers shown in panel a. (**c**) 2D gel electrophoresis and Western blot of concentrated supernatant from *Angptl4* – HEK293 stable cell line incubated with ManNAc or control, and analyzed for Angptl4 expression and binding with sialic acid binding lectin MAA. Green arrow and line highlight high pI protein in the control treated group, whereas blue arrow in the ManNAc treated group shows neutral pI protein. (**d**) Same study as panel c, except done with supernatant from the GEC stable cell line. (**e**) 2D gel electrophoresis and Western blot studies of glomerular protein from NPHS2-*Angptl4* transgenic rats given tap water or tap water with ManNAc for 12 days. Blots were analyzed for neutral pI (enclosed in red ovals) and high pI (enclosed in green ovals) Angptl4 using anti-Angptl4 antibody and sialic acid

binding lectin Sambucus Nigra (SNA I). (**f**) Percentage of neutral and high pI Angptl4 within each group as assessed by densitometry. (**g**) Albuminuria in NPHS2-*Angptl4* transgenic rats given tap water (Control group) or tap water with ManNAC (Treatment group) for 12 days (Treatment group, ManNAC phase), followed by plain tap water for 24 days (Treatment group, Washout phase). Values are expressed as a percentage of the baseline albuminuria (designated as 100%). Individual tracings are shown in Supplementary Fig. 8. In panel b, all * differences are with control values. In panel g, all * differences are with baseline values. Loading controls shown in Supplementary Fig. 6. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.